Isolation and Identification of Indole-3-Ethanol (Tryptophol) from Cucumber Seedlings[1,2]

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Summary. Crude ether extracts of green shoots of *Cucumis sativus* L. promoted the elongation of cucumber hypocotyl segments. Purification of the extract was accomplished by DEAE cellulose, silicic acid, and magnesium silicate chromatography followed by gel filtration and preparative thin layer chromatography. Identification of the growth promoter as indole-3-ethanol was achieved by mass spectrometry, thin layer and gas chromatography, and ultraviolet and visible spectroscopy, as well as by physiological characteristics.

The central role of indolic compounds as plant growth regulators is well established. However, few growth-promoting indolic compounds have been isolated in pure form from plant tissue. The most extensively studied is indole-3-acetic acid (IAA), which has been isolated in crystalline form from several sources (5, 10, 20). Jones et al. have isolated pure indoleacetonitrile from immature cabbage heads (8). Indoleacetaldehyde has been identified in plant tissue by Larsen (11). Evidence for the occurrence of other growth-promoting indolic compounds, such as indolepyruvic acid (18), indoleacetamide (1), the ethyl ester of IAA (19), and others, is based primarily on chromatographic behavior of crude extracts in 1 or 2 solvents. While chromatographic data are valuable, they are not conclusive, especially when used as the sole criteria with impure preparations.

The natural occurrence of indole-3-ethanol (IEt) in higher plants has not previously been demonstrated. Evidence based on paper chromatographic methods and color tests indicated that *Acetobacter xylinum* can produce IEt. It was proposed that IEt and IAA might arise from indoleacetaldehyde in this organism by a dismutation reaction (12). Kaper and Veldstra, using chromatographic techniques, have demonstrated IEt as a product of physiological origin from *Agrobacterium tumefaciens* (9). Bailey and Gordon reported the tentative identification of IEt in *Diplodia natalensis*

culture media containing tryptophan or tryptamine (2). Perley and Stowe observed IEt production from tryptophan by *Bacillus cereus* and by *Taphrina deformans* (15, 16). Wightman has reported some evidence for the occurrence of IEt in tomato seedlings after the application of tryptophan or indole-3-lactic acid through the cut stems (23). In this system it was postulated that indolelactic acid was decarboxylated to IEt which was then oxidized to IAA. Libbert and Brunn have presented chromatographic data showing that IEt was formed when the IAA-forming enzyme of peas was incubated for 12 hours in a tryptophan solution (14).

This paper offers evidence for the natural occurrence of IEt in cucumber shoots. Our method for the purification of IEt should be applicable to other neutral indolic compounds. A method is described for the bioassay of IEt using the cucumber hypocotyl.

Materials and Methods

Bioassay. Seeds of *Cucumis sativus* L. cv. National Pickling (W. Atlee Burpee Seed Co.) were soaked for 2 hours and sown in vermiculite (Terralite, Zonolite Co.) moistened with tap water. Seedlings were grown under constant illumination (640 ft-c) for 5 to 6 days in a growth chamber at 26 to 27°. Segments were cut so that the apical 2.5 cm of the hypocotyl was included as well as the still quiescent epicotyl. The cotyledons were removed in all cases. The segments were distributed in lots of 5 into Stender dishes containing 5 ml of the test medium. Dishes were returned to the growth chamber and kept under constant illumination throughout the incubation period. After 4 to 6 hours the segments were measured to the nearest 0.5 mm. Hypocotyl segments of *Cucurbita pepo* L. cv. Fordhook Zucchini Improved were used in a similar manner.

Extraction and Purification. Seeds of *C. sativus* were sown as described above and grown in the light (640 ft-c). After 7 to 8 days the shoots were harvested, weighed, and extracted with ethyl ether (approximately 2 liters per kg fr wt tissue) for 2 to 3 hours at room temperature. After decantation of the first extract, the tissue was ground in a meat grinder and extracted twice more with fresh ethyl ether. The combined ether fractions were separated from the cellular water and evaporated to dryness. The gummy green residue was resuspended in double distilled water (250 ml per kg fr wt tissue) and filtered through a 4 cm pad of DEAE-cellulose. This operation ridded the extract of pigments. DEAE-cellulose has the further advantage of binding IAA. The growth-promoting filtrate was extracted 5 times with equal volumes of ethyl ether. The combined ether fractions were evaporated and the water fraction discarded. Several 5 to 15 kg batches of tissue were extracted, processed as described above, and stored at 4° until a total of 93 kg of tissue had been extracted. At this time, the residues from each batch were combined to yield 3.21 g of a yellow oil. The combined residues were dissolved in a minimal amount of petroleum ether (b.p. 30 to 60°) : ethyl ether (3:1, v/v) and placed on a silicic acid column (2.9 × 25 cm). The column was eluted in a stepwise fashion using increasing concentrations of ethyl ether in petroleum ether according to Method A of Hirsch and Ahrens (7). The growth promoting fractions from this step as well as all subsequent steps were determined by removing aliquots of the various fractions, evaporating the solvent, and dissolving the residue in 5 ml of water which was then bioassayed. Activity was observed in the 100 % ethyl ether fractions. The active fractions from the silicic acid column were combined and evaporated to dryness. The residue (117 mg) was suspended in a minimal amount of CCl_4 : $CHCl_3$ (3:1, v/v) and placed on a 2.0 × 20 cm magnesium silicate M-1 (Bio-Rad) column packed in CCl_4. The column was eluted in a stepwise pattern using increasing concentrations (0, 10, 20, 30, 40 and 50 %, v/v) of $CHCl_3$ in CCl_4. At each step, 100 ml of solvent was used. Finally, the column was eluted with 250 ml of 100 % $CHCl_3$. The 100 % $CHCl_3$ fractions contained the growth-active substance. Further purification was obtained by evaporating the active fractions to dryness and dissolving the residue (33 mg) in a minimal amount of absolute methanol. The methanol solution was placed on a Sephadex LH-20 column (2.5 × 75 cm) and eluted with methanol. Ten-ml fractions were collected using an automatic fraction collector. Selected fractions were assayed for biological activity and for absorption at 280 nm (fig 1). The active fractions were evaporated in a rotary flash evaporator leaving 3.5 mg of residue which was dissolved in ethyl ether and streaked on an Eastman Chromagram sheet (Type K 301 R2). The sheet
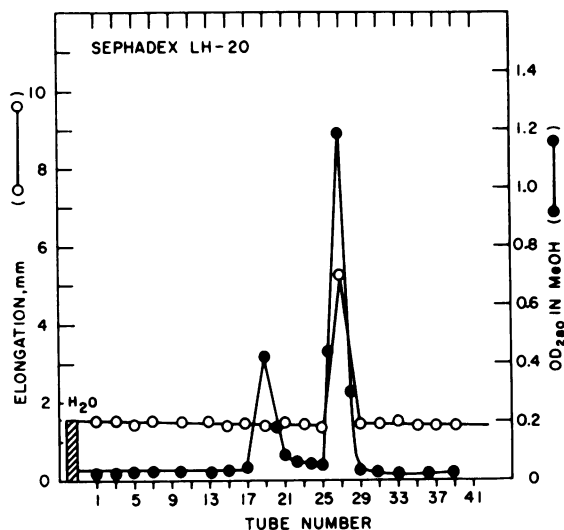

Fig. 1. Elution of cucumber factor from Sephadex LH-20 by methanol. 10-ml Fractions collected. Selected fractions assayed for biological activity (4 hr incubation) and for absorption at 280 nm.

was clamped between 2 Eastman Chromagram chamber plates and developed with 100 % $CHCl_3$. The chromatogram was divided into several zones and the silica gel scraped off and eluted with anhydrous ethyl ether. An aliquot of the R_F 0.15 to 0.3 eluate was active in the bioassay. The active eluate was evaporated under nitrogen yielding 2.7 mg of material. Final purification was achieved by rechromatography on Sephadex LH-20 as described above. The active fractions were combined and evaporated under nitrogen leaving 2.5 mg of
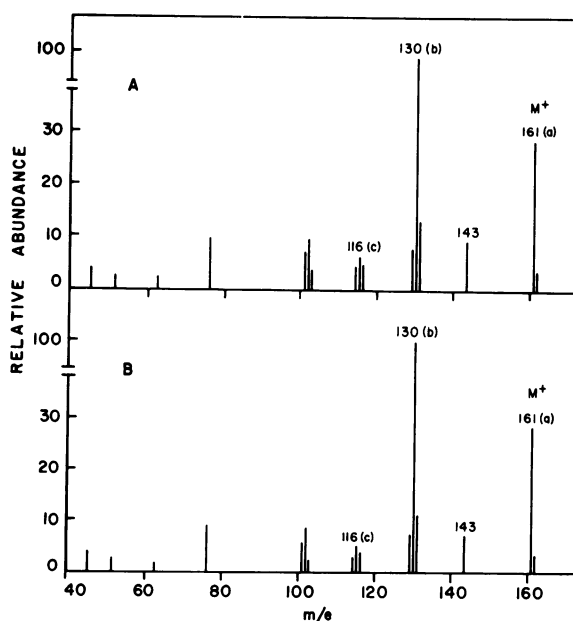

Fig. 2. Mass spectra. A) Cucumber factor. B) Indole-3-ethanol.

a light yellow gum. The progress of the purification was followed by removing an aliquot of the active material after each step in the purification procedure and spot-chromatographing the residue on silica gel in several solvents. Organic compounds were detected by exposing the dry chromatogram to iodine vapor.

The purity of the final preparation was checked by thin layer chromatography in 5 solvents of widely differing polarity: ethyl ether : hexane (1:1, v/v), isopropanol : NH_4OH : H_2O (85:5:15, v/v), $CHCl_3$: 96 % acetic acid (95:5, v/v), 100 % $CHCl_3$, and $CHCl_3$: methanol : glacial acetic acid (75:20:5, v/v). Detection of spots with iodine vapor and with Ehrlich's reagent (2 %, w/v p-dimethylaminobenzaldehyde in 2 N HCl in 80 % ethanol) showed the final preparation to be homogeneous in each of these solvents.

Results

A sample of the growth promoting cucumber factor was subjected to mass spectrometric analysis using an A. E. I. MS-9 mass spectrometer (fig 2A). A strong parent ion peak is seen at m/e 161. This value is consistent with the empirical formula $C_{10}H_{11}NO$. The main fragmentation pattern of the parent ion is rationalized as follows:

a) m/e = 161 b) m/e = 130 c) m/e = 116
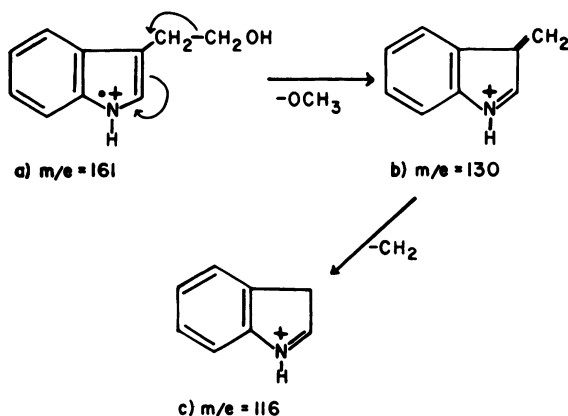
This fragmentation pattern is characteristic of simple indoles and can be readily visualized if one assumes that the first electron to be removed originates from the indole nitrogen. This characteristic was of value in the structural identification of 5-methoxyindole-3-acetic acid isolated from bovine pineal glands (13). A secondary fragmentation pathway of cucumber factor appears to be the loss of water from the molecular ion resulting in the peak at m/e 143. Metastable peaks were seen at m/e 81.5 and 126. The mass spectrum of the factor was compared with one obtained using a known sample of indole-3-ethanol (K & K Laboratories). Prior to analysis, the commercial IEt was purified by thin layer chromatography on silica gel using 100 % $CHCl_3$ as the mobile phase and then recrystallized twice from benzene : petroleum ether. The spectrum obtained for IEt (fig 2B) was similar in every respect to that obtained with the unknown growth promoter from cucumbers.

IEt and the cucumber factor were compared by thin layer chromatography (Eastman Chromagram sheets) using 10 different solvent systems. Compounds were detected by spraying with Ehrlich's reagent and subsequent development at 80° for 10 minutes. The R_F values were calculated and the Ehrlich reaction colors noted (table I). Cucumber factor and IEt were also compared by gas chromatography, using an Aerograph Model A-90-P3 (column: 20 % SE-30 on Chromosorb W 60/80, 5 ft × 0.25 in, column temp 210°, detector temp 280°, injector temp 280°, carrier gas helium at 60 ml/min). Under the conditions employed, retention times of 6.94 minutes were recorded for both IEt and the cucumber factor. In contrast, the ethyl ester of IAA had a retention time of 10.51 minutes. Dedio and Zalik have recently separated certain volatile indoles including IEt using similar conditions (3).

Figure 3 shows the ultraviolet absorption spectra of IEt and the cucumber factor. Absorption maxima appeared at 274, 281, and 291 nm in both cases. The spectrum of IAA is included for comparison. Harbo and Aasheim used the visible

Table I. R_F *Values for Cucumber Factor and Indoleethanol in Silica Gel Thin Layer Chromatography*
Eastman Chromagram sheets were used for these determinations.

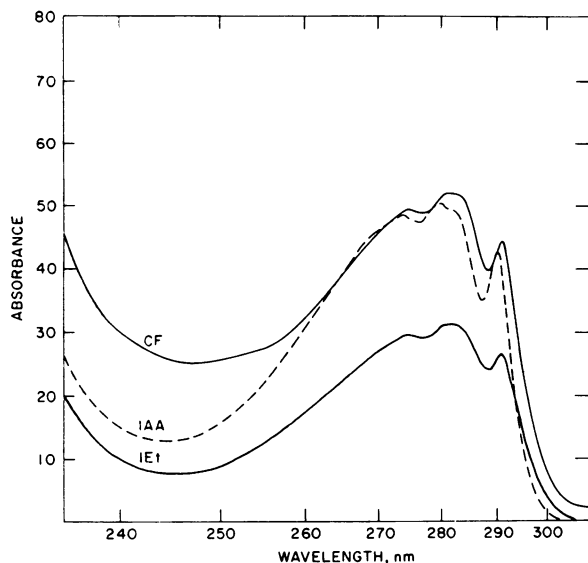| Solvent	Cucumber factor	Indoleethanol
	R_F	R_F
Hexane : ethyl ether (1:1, v/v)	0.18	0.20
Hexane : ethyl ether (1:4, v/v)	0.59	0.62
Isopropanol : NH_4OH : H_2O (10:1:1, v/v)	0.99	0.99
$CHCl_3$: methanol : glacial acetic acid (75:20:5, v/v)	0.98	0.99
$CHCl_3$: methanol : CCl_4 (50:25:25, v/v)	0.81	0.80
$CHCl_3$: methanol : CCl_4 (5:4:4, v/v)	0.99	0.99
$CHCl_3$	0.20	0.20
CH_2Cl_2	0.69	0.70
1-Butanol : ethanol : H_2O (40:4:19, v/v)	0.92	0.92
Ethanol : CCl_4 (1:1, v/v)	0.99	0.99

FIG. 3. Ultraviolet absorption spectra of cucumber factor, indole-3-ethanol, and indole-3-acetic acid in anhydrous ethyl ether. Concentrations of cucumber factor and indoleacetic acid adjusted to give approximately equal absorbance. Concentration of indoleethanol adjusted for lower absorbance to facilitate comparison.

spectrum of the colored products of the action of Ehrlich's reagent on IEt as evidence for its occurrence in culture media of *Acetobacter xylinum* (6). We compared the visible spectra of cucumber factor and of IEt after the addition of Ehrlich's reagent. The spectra were identical, showing sharp peaks at 575 and 545 nm with a small peak at 460 nm and a large, broad peak from 395 to 420 nm with a maximum at about 405 nm. Infrared and nuclear magnetic resonance spectra of small samples of the cucumber factor were also obtained. These corresponded to those of known IEt; how-

ever, the test samples were so small as to give spectra unsatisfactory for publication.

Dose-response curves for the factor and IEt in the cucumber hypocotyl test are given in figure 4. The response to optimal IAA is shown for comparison. In our preliminary studies of the factor it was noted that, while some species responded strongly, others responded weakly or not at all. Zucchini squash was one of the species that did not respond significantly to the factor. IEt was also inactive in the squash hypocotyl test (fig 5).

FIG. 5. Dose-response curve for indole-3-ethanol in the squash hypocotyl test. Measurements made after 6 hours. Bar indicates segment growth in 0.1 mM IAA.

Discussion

This paper presents convincing evidence for the occurrence of IEt in cucumber seedlings. The mass spectra of IEt and the naturally occurring product are identical. The factor and IEt cochromatogram on silica gel in 10 solvents of widely differing polarity and have identical retention times in gas chromatography. The ultraviolet absorption spectra of the factor and IEt are identical as are the visible spectra of the colored products of reactions with Ehrlich's reagent. The cucumber factor and IEt elicit similar growth responses in the cucumber hypocotyl test and both are inactive in the squash hypocotyl test.

The structure indole-3-ethanol is favored over indole-2-ethanol. Various 3-substituted indoles such as IAA are known to occur in plants, while no likely precursor of indole-2-ethanol is known. The physiological response caused by the factor is identical with that caused by known indole-3-ethanol. The reaction with Ehrlich's reagent also suggests the structure indole-3-ethanol, since the Ehrlich test is characteristic of indoles in which the 2-position is unsubstituted (4).
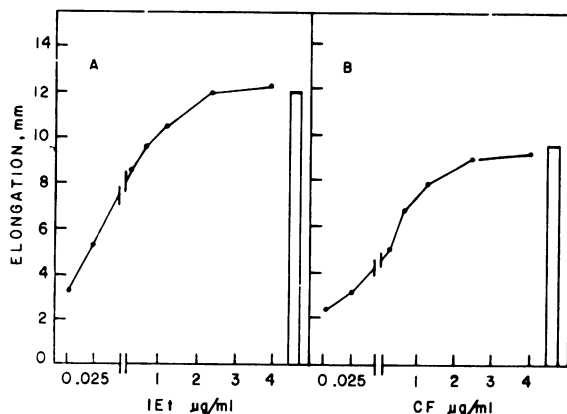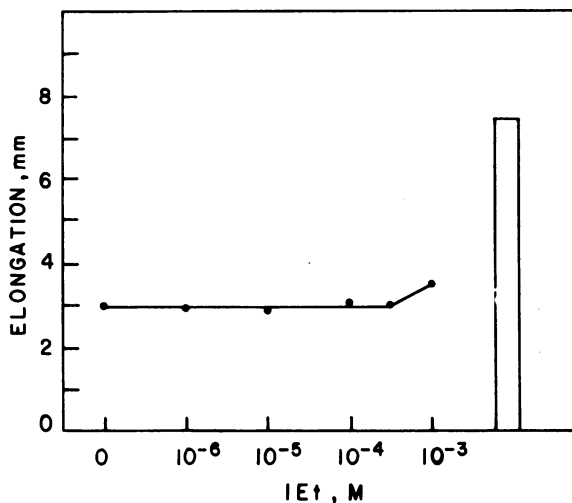

FIG. 4. Dose-response curves in the cucumber hypocotyl test. A) Indole-3-ethanol. B) Cucumber factor. Measurements made after 20 hours incubation. Bars indicate segment growth after 20 hours in optimal 0.1 mM IAA. Experiments A and B performed on different days.

IEt has been reported to be slightly active in the *Avena* and pea tests (21) and moderately active in the wheat cylinder test (22). However, the alcohol analogs of the known acidic auxins have, in general, been very little investigated. Interestingly, IEt is as active as IAA in the cucumber hypocotyl assay. We have found IEt to be an active growth promoter in certain species other than the cucumber. These data will be reported separately.

One may ask whether IEt is itself an active growth hormone or whether it is converted to IAA before it is active. We are not yet prepared to answer this question; however, a 2-step oxidation of IEt to IAA via indoleacetaldehyde seems likely. The interaction of IEt with IAA in the cucumber assay is consistent with this view (17). This question is currently under investigation.

Acknowledgments

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